

IDENTIFICATION AND STABILITY OF ACYLATED ANTHOCYANINS IN PURPLE-
FLESHED SWEETPOTATO p40

by

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Abstract

We previously selected a purple-fleshed sweetpotato p40 clone that has been shown to protect against colorectal cancer in a murine model. This study is to identify anthocyanins by using HPLC/MS-MS and assess the stability during various coking conditions. P40 possesses a high content of anthocyanins up to 13 mg/g dry matter. Total 12 acylated anthocyanins with caffeic, ferulic, and p-hydrobenzoic acid have been identified on either cyanidin or peonidin bases. The top three major anthocyanins are cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside, peonidin 3-caffeoyl sophoroside-5-glucoside, and cyanidin 3-(6''-caffeoyl-6''-feruloylsophoroside)-5-glucoside, which account for an half of the total anthocyanin contents. Seven non-, mono-, or di-acylated cyanidin species and five mono- or di-acylated peonidin species contribute for 69% and 31% of total anthocyanins, respectively. Over 80% of total anthocyanins measured by acid hydrolysis were cyanidin derivatives. Therefore, as a cyanidin-predominated variety, p40 is unique when compared with other reported purple-fleshed sweetpotatoes that usually contain more peonidin than cyanidin. While baking does not impact overall contents of anthocyanins, steaming, high pressure cooking, microwaving, and frying significantly reduce 20% of total anthocyanin contents. Mono-acylated anthocyanins show a higher resistance against heat than di- and non-acylated. Among of which, cyaniding 3-p-hydroxybenzoylsophoroside-5-glucoside exhibits the best thermal stability. Better understanding of dietary anthocyanins and their stabilities may lead to the development of a functional anthocyanin-enriched sweetpotato product for health benefits.

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Chapter 1 - Introduction

The color of a food affects the consumer's choice and oftentimes adds a value to the food. Vivid natural colors are associated with perceptions of being fresh and healthy and lead to willingness of purchase and increased appetite. Natural pigments of plant origins--such as chlorophyll, anthocyanins, and carotenoids--add not only colors to the food, but also potential health benefits to the consumers (Delgado-Vargas, Jiménez, & Paredes-López, 2000; He & Giusti, 2010). Plants cultivated to bear different colors -- for example, purple corn, red cabbage, blood orange, and black rice -- are considered novelties in many parts of the world and possess higher values in nutrition and economy than their conventional counterparts.

In today's market, many vegetables and fruits have been tailored toward purple and blue hues, which are essentially contributed by the enrichment of anthocyanins. Anthocyanins are a group of hydrophilic plant pigments that are naturally occurring in most berries and vascular plants and responsible for purple and blue colors. Anthocyanins were officially approved as dietary additives based upon generally recognized as safe (CFR - Code of Federal Regulations Title 21) and were heavily utilized as natural colorants due to their color intensity, non-toxicity, and excellent water solubility (He & Giusti, 2010). Comparing with artificial food colors and dyes, berry anthocyanins are usually unstable and susceptible to degradation caused by extrinsic and intrinsic factors. To expand the application of anthocyanins to more diverse food forms, stabilized anthocyanins must be employed including acylated anthocyanins from purple-fleshed sweet potato (PSP) (Goda et al., 1997).

The sweet potato, as one of the most important staple crop in some parts of Africa and Asia, has been bred to synthesized anthocyanins. Anthocyanin-enriched sweet potatoes carry

intense purple/red color in the flesh, and in some cases, the skin. The Purple-fleshed sweet potato (PSP) supplies all the macronutrients of a regular sweet potato and, in addition, twice of the anti-oxidation activity (Lim et al., 2013). More particularly, PSP is a unique crop that contains acylated anthocyanins that seem stable. Considering the necessity of cooking process, the heat stability of anthocyanins is particularly important (Goda et al., 1997; Steed & Truong, 2008).

In this study, we selected a new variety of purple-fleshed sweet potato, clone p40, from a population of seeds obtained from the International Potato Center in Lima, Peru. In addition to a high content of total phenolics and antioxidant capacity, p40 possesses a high content of acylated anthocyanins. We hypothesized that acylated anthocyanin-enriched p40 may provide a pronounced stability during cooking condition. To test this hypothesis two studies were performed: identification and thermal stability of PSP anthocyanins, were elucidated.

Chapter 2 - Literature Review

Anthocyanins as natural pigments

Anthocyanins are known as the largest group of water-soluble natural pigments in plants. Anthocyanins are widely distributed in nature, primarily responsible for pigmentations of orange, red, blue, and purple. Accumulating in leaves and flowers, anthocyanins provide protections for plant against ultraviolet damages and also play an important role in attracting insects for better pollination. Occurrences of anthocyanins are also abundant in fruits and rhizomes, as well as other plant tissues commonly consumed by human beings (He, 2008).

Anthocyanins are nature colorants used in beverage industries to impart intense colors. In Asian countries, anthocyanin-based colorants have been widely used in baking and brewing products as well. Anthocyanins have received more attention recently due to a raised demand for natural and healthy additives, while artificial and insect-based colorants are criticized for adverse health effects. For example, a recent study reported the cochineal extract, a red dye used in cocktails, red pickles, and candies, was found to induce immediate allergy-like face swelling and asthma (Yamakawa, Oosuna, Yamakawa, Aihara, & Ikezawa, 2009). Another study found that the per capita consumption of artificial food colorings quadrupling in the last 50 years might cause hyperactivity in some children populations (Arnold, Lofthouse, & Hurt, 2012). Development of natural anthocyanin as food colors may help to reduce use of artificial colorants.

Occurrence and structure of anthocyanins

Berry fruits and purple-colored vegetables are primary sources of edible anthocyanins. The occurrences of anthocyanins vary greatly among different plants and cultivars. The most known and available anthocyanin-containing fruits to the general public are grapes, cherries,

blueberries, and strawberries. Eggplant is the only common vegetable that contains high anthocyanin; other specifically bred vegetables such as red cabbage and radish are good sources of anthocyanin as well, but are consumed less frequently (Clifford, 2000; Horbowicz, Kosson, Grzesiuk, & Dębski, 2008).

Anthocyanins are derivatives of anthocyanidins; polyphenolic secondary metabolites of plants belong to flavonoids. There were 21 anthocyanidins found in plants and the top six abundant anthocyanidins are cyanidin (30%), delphinidin (22%), pelargonidin (18%), peonidin (7.5%), malvidin (7.5%), and petunidin (5%) (Figure 1) (Andersen & Markham, 2010; Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2004).

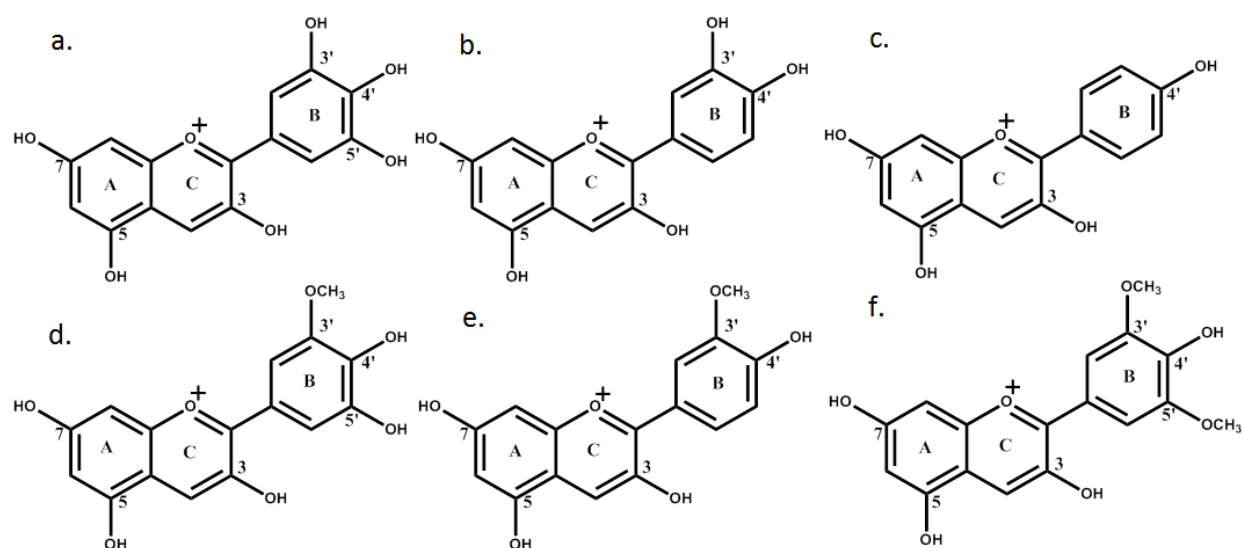


Figure 1. Chemical structures of the top six anthocyanidins (anthocyanin aglycons) a: delphinidin; b: cyanidin; c: pelargonidin; d: petunidin; e: peonidin; f: malvidin.

Pulling together a vast of literature, over 600 anthocyanins were identified deriving from various anthocyanidin bases (He & Giusti, 2010), where cyanidin 3-glucoside was the most abundant derivative that account for nearly half of anthocyanins (Clifford, 2000). The anthocyanidin molecule possesses eight conjugated double bonds as well as a positively charged

pyran ring to act as light-absorbing chromophores. The main differences between anthocyanins exist in three ways: aglycone structure, glycosylation, and acylation moieties. It is very rare that natural anthocyanins exist in aglycon form. Oftentimes hexose and pentose are jointed to anthocyanidins at C3, C5, or both positions. Glycosylations improve stability and hydrophilicity of anthocyanins; common glycosides include glucose, rhamnose, galactose, arabinose, xylose, rutinose. In addition to glycosylation, cinnamic acids and aliphatic acids are attached to glycosides in some specially bred tuber crops, red cabbage, and certain grapes, (Figure 2) (Giusti & Wrolstad, 2003). The occurrence of natural acylated anthocyanins was limited to certain food resources as shown in Table 1.

Table 1. Sources of acylated anthocyanins (re-edited from Giusti & Wrolstad, 2003)

Source	Pigment composition
Radish (<i>Raphanus sativus</i>)	Pelargonidin derivative acylated with one cinnamic acid and an aliphatic acid
Potato (<i>S. tuberosum</i>)	Pelargonidin derivatives acylated with one cinnamic acid
Black carrot (<i>Daucus carota</i> L.)	Cyanidin derivatives acylated with one cinnamic acid
Red cabbage (<i>Brassica oleracea</i>)	Cyanidin derivatives acylated with one or two cinnamic acids
Grape (<i>Vitis labrusca</i>)	A mixture of five different aglycons, acylated and non-acylated with p-coumaric acid
Sweetpotato (<i>Ipomoea batatas</i>)	Cyanidin or peonidin derivatives acylated with one or two cinnamic acid

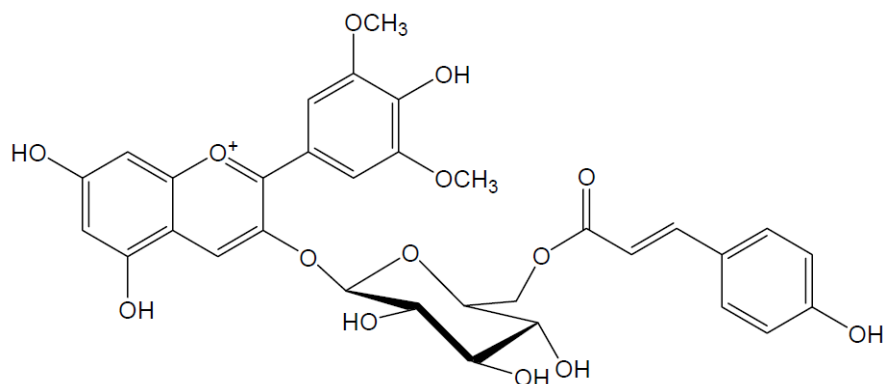


Figure 2. Chemical structure of an acylated anthocyanin (malvidin-3-p-coumaroyl glucoside) in grape skin (re-printed from He, 2008)

Stability of anthocyanins

As a natural water-soluble pigment, anthocyanins are known to be unstable in certain conditions. In fact, the stability of anthocyanins, especially in neutral to alkaline environment, has become a hurdle to apply anthocyanins in many food forms. A number of factors can deter anthocyanin stability such as pH, heat, oxygen, light, enzymes, moisture etc. This section discusses the major environmental impacts and possible modifications to improve anthocyanin stability.

pH and anthocyanins

pH is a well-known factor, affecting anthocyanins. The most notable phenomenon of anthocyanins in a pH gradient is the change of color. In fact, anthocyanins were used as crude pH indicators in early stage science (Forster, 1978). As shown in Figure 3, anthocyanins exist as flavylium cations when pH is lower than 3, carrying oxygen by the positive charge. Rapid deprotonation occurs at oxygen and skeleton hydroxyl groups when pH increases to 3-5, transforming the flavylium cation to quinonoidal bases and shifting light absorbance away from ~520 nm to a blue/violet hue (Wrolstad, Durst, & Lee, 2005). In alkaline condition, quinonoidal bases are further deprotonated and form quinonoid anions. Alternatively, anthocyanins form colorless carbinol pseudobase and light yellow chalcones through hydration reactions at C-2 position in slightly acidic environment, which result in color fading. Noteworthy, the formation of flavylium to carbinol pseudobase are rather rapid but reversible upon acidification (Brouillard & Delaporte, 1977; He, 2008).

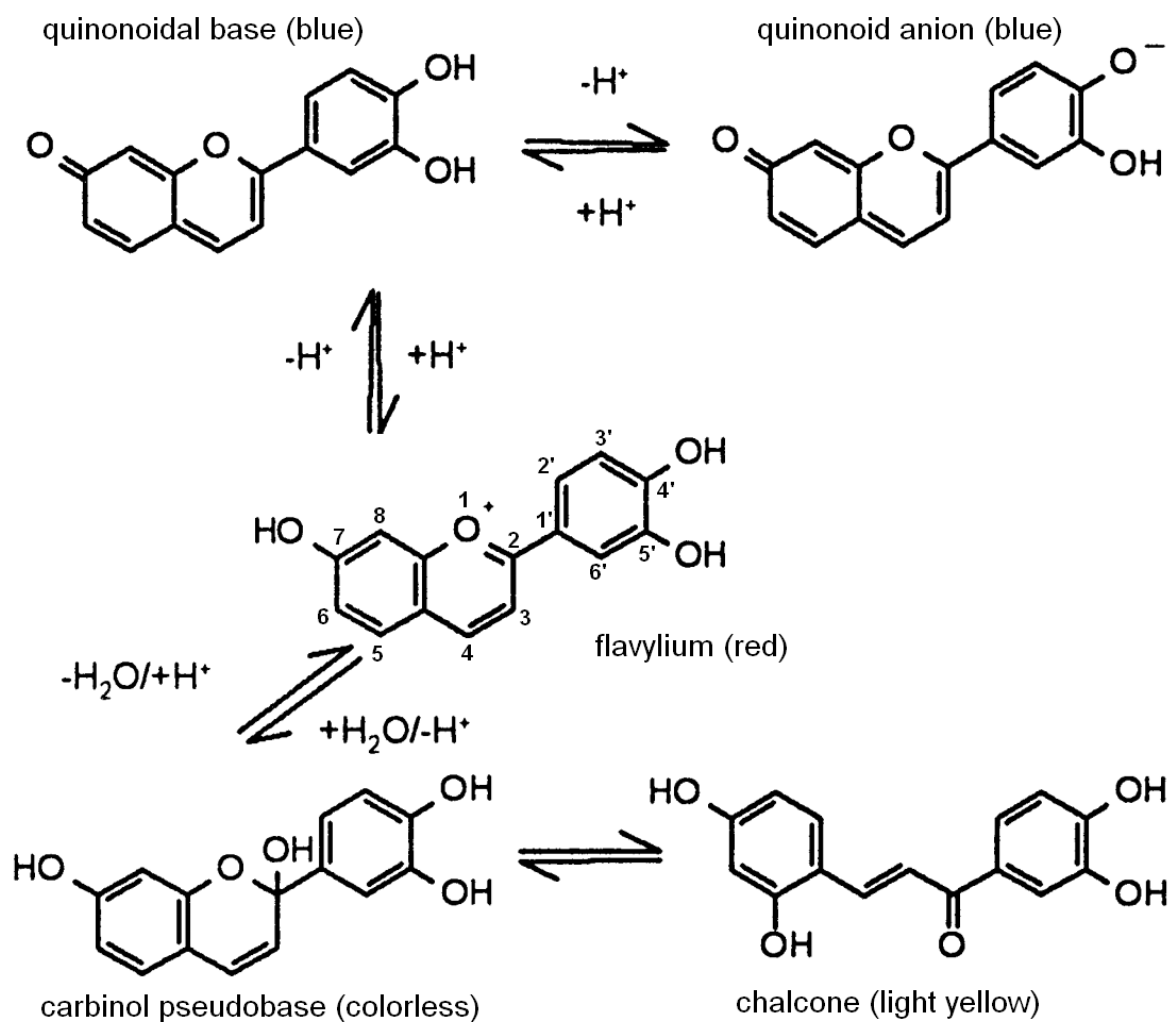


Figure 3. pH-dependent transformation of anthocyanidin (modified according to Kähkönen & Heinonen, 2003)

Thermal stability of anthocyanins

Anthocyanins are thermal liable compounds. Heat introduced in processing and storage plays an important role in accelerating transformation to chalcones, and eventually decomposing anthocyanins to benzoic acid derivatives, resulting in color loss. In general, refrigeration and acidic pH are critical conditions to preserve anthocyanin-containing materials. Using strawberry extract as an anthocyanin source that contains mainly pelargonidin-3-glucoside, a systematic study reported the half-life of total anthocyanins was 3.2 h when heated at 95 °C in pH 1 aqueous

environment. A majority of pelargonidin-3-glucoside was broken down to pelargonidin and further converted to colorless forms (Sadilova, Stintzing, & Carle, 2006). The same study also evaluated elderberry and black-carrot extracts and indicated their half-lives were 1.9 h and 4.1 h, respectively. Storage temperature places another hurdle in commercializing anthocyanin-based colorant. Floral anthocyanin used in carbonated beverage model for 180 days degraded by 90% and 30% at room temperature and 4 °C, respectively (Amr & Al-Tamimi, 2007).

Acylation remarkably improves stability of anthocyanins by retarding chalcone formation through co-pigmentation. Purple-fleshed sweetpotato extract, as a critical di-acylated anthocyanin source, was reported having superior color stability in various food models (Steed & Truong, 2008). The stabilizing mechanism of intramolecular co-pigmentation with acyl groups is further discussed below.

Co-pigmentation and color enhancement

Stabilization of anthocyanins can be achieved by co-pigmentation, a phenomenon in which pigments associate with other compounds (co-pigments) and result in a color enhancement or shift, by blocking chromophores from hydration (Figure 4) (Dangles, Saito, & Brouillard, 1993; Davies & Mazza, 2002). Co-pigmentation phenomenon was brought to attention early in 1915 where the color of malvidin 3-glucoside was intensified by tannin addition (Boulton, 2001). It is accepted that co-pigmentation occurs in different ways depending on the nature of co-pigments. Formations of intermolecular and intramolecular compounds were known important means strengthen and shift colors of anthocyanins.

Co-pigments, such as phenolic acids and flavonoids, exhibit protective effect to anthocyanins by forming H-bonds and hydrophobic interactions. Hence on the accompanied anthocyanin molecule, the previously vulnerable carbon at position 2 is protected against

nucleophilic attack from water and thus prevent the formation of carbinol pseudobase. The complex formed between anthocyanins and non-colored molecules was recognized as intermolecular co-pigmentation (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Malien-Aubert, Dangles, & Amiot, 2001). Intermolecular copigmentation occurs to every species of anthocyanin and results in bathochromic shift, i.e. maximum absorption shift to higher wavelength, causing a blue hue, or hyperchromic effect that fortifying the original color; however, the concentration of anthocyanins and co-pigments must reach a relatively high level (~5 mM) to observe significant color enhancement (Asen, Stewart, & Norris, 1972). Acylated anthocyanins can be viewed as covalently bound co-pigmented anthocyanins. Aromatic residues of acyl groups fold back and stack on anthocyanin skeletons, and provide protections to flavylum containing chromophores intra-molecularly. Sandwich-like models, where acyl groups overlap on both side of anthocyanins, are suggested for the stabilizing mechanism in di-acylated anthocyanins. It is reported that acylated anthocyanins are more stable than non-acylated anthocyanins due to the strength of covalent bonds, and di-acylation provides more complete stabilizing power than mono-acylation (Brouillard, 1981).

Self-association takes place when co-pigment is formed. Boulton, (2001) reported anthocyanin solutions disobey Beer's law when it is concentrated or diluted in constant pH. For example, cyanidin 3-5-diglucoside at 5 mM displayed 200 times intensification as of 50 μ M (twice the absorbance as expected) (Rein, 2005). Multivalent metals, typically, Fe^{3+} , Cu^{2+} , Mg^{2+} , and Al^{3+} joint and stabilize anthocyanin molecules. However, metal complexations tend to stabilize quinoidal bases and give blue hues; therefore, anthocyanin-metal complexes are less interested in food industries.

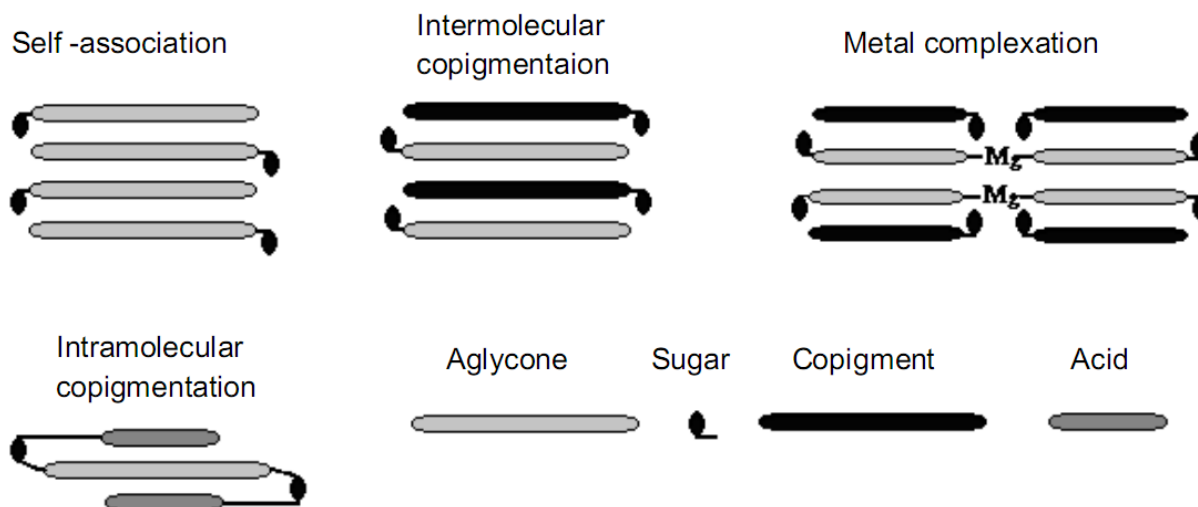


Figure 4. Copigmentation mechanisms (re-printed from Rein, 2005)

Analytical methods for anthocyanins

Two categories of analytical methods have been applied in anthocyanin studies. A conventional and rapid technique, i.e., pH differential colorimetric method by spectrophotometry, was used for many decades to determine total monomeric anthocyanin content. It was based on the reaction of flavylum to carbinol pseudobase, in which maximum absorbance around 520 nm diminishes as pH increases. The difference of absorbance between pH 1 and 4.5 was oftentimes used and expressed in following formula, excluding interferences from other phenolic acids of absorbance at 700 nm; the total anthocyanin contents were then corrected based upon a known standard (Horbowicz et al., 2008).

$$A = (A_{\lambda_{\max}} - A_{700})_{\text{pH}1.0} - (A_{\lambda_{\max}} - A_{700})_{\text{pH}4.5}$$

Another method is reversed-phase high performance liquid chromatography (HPLC) coupled with photodiode array detector (PDA), which has become the most common qualitative and quantitative method for anthocyanins. HPLC-PDA is more accurate, sensitive, and

repeatable than pH differential method. Identification of each compound, however, requires the corresponding standard, which sometimes is difficult to obtain. Lee, Rennaker, & Wrolstad, 2008, studied correlation of anthocyanin quantification between pH differential method and HPLC method on a total of 517 anthocyanin samples, and reported that HPLC method and pH differential method were well correlated ($R=0.931$) although HPLC method tended to give a slightly higher value (Lee et al., 2008). A recent technique of combination of HPLC with mass spectrometry detector (MS) has been employed in anthocyanin studies to obtain precise molecular traits of each peak while using a minimum number of the standards (Giusti, Rodríguez-Saona, Griffin, & Wrolstad, 1999; Kim et al., 2012; Tian, Giusti, Stoner, & Schwartz, 2005; Tian, Konczak, & Schwartz, 2005; Truong et al., 2010).

Bioavailability and Disease prevention of anthocyanins

Anthocyanins are bioavailable compounds (Kong, Chia, Goh, Chia, & Brouillard, 2003), and readily to be absorbed in both stomach and small intestine. A study that utilized stomach gavage in a rat model demonstrated that grape anthocyanin malvidin-3-glucoside was detectable in the plasma within 6 min by HPLC/MS, presenting a solid evidence of stomach absorption (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2003; Talavéra et al., 2003). The small intestine as well has been proven as an absorption site of anthocyanins. Demonstrated by an intestinal perfusion study in anesthetized rats, 10-22% of anthocyanins disappeared after trickling through the small intestine under facilitation of saline buffer (Talavéra et al., 2004). Comparing with berry anthocyanins, acylated anthocyanins in PSP origin have received much less attention, probably due to berry anthocyanins being less expensive and thus are favored in industry usage. Because of the advanced breeding technologies, anthocyanins in PSP have been improved drastically in terms of quality and quantity. PSP is becoming a promised functional food in the

market, providing some advantages of superior stability and comparable bioavailability to berry anthocyanins. For example, studies have demonstrated peonidin 3-caffeoyl sophoroside-5-glucoside, a high molecular weight mono-acylated anthocyanin, was absorbed as well as non-acylated anthocyanins with a plasma concentration up to 50 nM at 30 min and diminished at 120 min post oral administration at a dose of 38.9 $\mu\text{mol/kg}$ in mice (34). The t_{max} and $t_{1/2}$ value of peonidin 3-caffeoyl sophoroside-5-glucoside was similar to cyanidin 3-glucoside, while other di-acylated anthocyanins, such as peonidin 3-(6",6" ' -dicaffeoyl sophoroside)-5- glucoside and peonidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside were also absorbed and detected in plasma within 30 min. Plasma antioxidant capacity, expressed by trolox equivalence, was elevated 1.5-folds at 30 min post administration (Suda et al., 2002). Epidemiologically, the consumption of anthocyanins has been related to low occurrence of certain diseases particularly colorectal cancer (Delgado-Vargas et al., 2000). Using a colon cancer-induced rat model a recent study showed grape and berry extracts significantly decreased fecal bile acid content and down-regulated the expression of cyclooxygenase-2, an enzyme responsible for inflammation and pain (Lala et al., 2006). Unabsorbed anthocyanin seemed to have a positive impact to colorectal health through the antioxidation mechanism (Lala et al., 2006). A newly published study from Lim et al., (2013) demonstrated that the number of azoxymethane (AOM) induced aberrant crypt foci in mice's colon decreased over 50% in the group fed with PSP containing diet.

Purple sweet potato anthocyanins

Purple sweet potatoes have gained popularity worldwide, mainly as a source to produce food colorants. In many countries such as South Korea, PSP is also eaten in raw, baked, and steamed forms (Kim et al., 2011). PSP anthocyanins have unique advantages over red cabbage and radish anthocyanins because the latter often carry unpleasant characteristic odors, and

therefore require additional deodorization process before adding to foods (Giusti & Wrolstad, 2003). Anthocyanin extract produced from PSP does not carry off flavors, and thus can be added at a relatively high amount to preserve color stability through self-association (Giusti & Wrolstad, 2003). Like other natural colors, anthocyanin-based colorants are more expensive than synthetic colors such as FD&C red #40 and indigo blue. Because of this it is very pertinent to research toward PSP with high anthocyanin content as it significantly lowers production cost. This thesis was necessitated as it provided a detail anthocyanin profiling and quantitative evaluation on a new PSP variety that appeared dark black-purple in its flesh. Furthermore, this work included a study to evaluate anthocyanin stability through multiply cooking methods and intended to find stable anthocyanins. The result of this work will finger print anthocyanins in the new PSP variety, and will ultimately benefit future PSP breeding.

Chapter 3 - Anthocyanin Characterization and Changes during Thermal Processing

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Introduction

Sweetpotato (*Ipomoea batatas*) is known as an excellent source of β -carotene (precursor of vitamin A), vitamin Bs, dietary fiber, minerals, and polysaccharides. In year 2003, the global production of sweet potato was estimated about 122 million metric tons, which was heavily consumed in rural part of China and western African countries (Wu et al., 2008). Purple-fleshed Sweet Potato (PSP) contains significant amount of anthocyanin than ordinary orange-fleshed sweet potato. Through years, a number of PSP varieties with different anthocyanin contents and profiles were increasingly bred and grown for their potential health benefits.

Differing from anthocyanins found in fruits and berries, PSP anthocyanins primarily exist as acylated forms. Acylation with various organic acids makes PSP anthocyanins unique and also means advantages in pH and heat resistances, light sensitivity, and overall stability. From a nutritional viewpoint, acylated anthocyanins have been reported to possess elevated activity in antioxidants and anti-mutagenicity (Suda et al., 2002). Biological activities of specific acyl groups are still under evaluation, however, it's believed that additional free phenolic hydroxyl groups may raise bio-functionality of anthocyanins. Among six common anthocyanidins, peonidin and cyanidin were found abundantly in PSP. Pelargonidin was a minor anthocyanidin found in a few varieties of PSP such as NC415 and Ayamurasaki with negligible content (Giusti et al., 1999; H. W. Kim et al., 2012; Truong et al., 2010). PSP as an acylated anthocyanin source has shown excellent coloring properties in numerous acidic to neutral foods that close to the synthetic FD&C red #40 (Suda et al., 2003). In Japan, PSP puree is a popular natural colorant and functional ingredient in the bakery, confectionery, juices, beverages, and dairy food industries (Dyrby, Westergaard, & Stapelfeldt, 2001; M. Giusti & Wrolstad, 2003; Suda et al., 2003). In order to be used as an additive, PSP is usually transformed to cooked puree, dried, and

powdered; those processes lead to pigment degradation along thermal treatments and oxygen exposure (Steed & Truong, 2008). Therefore, thermal stability of anthocyanin is recognized as a key property that affects overall quality.

The p40 is a variety of anthocyanin-rich sweet potato cultured at the John C. Pair Horticulture Research Center, Wichita, KS, by selecting from a large number of parent-seedlings provided by the International Potato Center in Lima, Peru. Lim et al., (2013) reported p40 extracts at 10-40 μ M significantly slowed growth of SW480 (human colon cancer) cells *in vitro* at G1 phase. Mice consumed p40 containing diet was less susceptible to azoxymethane-induced colorectal cancer in terms of aberrant crypt foci formation (Lim et al., 2013). The objective of this follow up study is to characterize anthocyanin composition of p40 quantitatively and qualitatively. High-Performance Liquid Chromatography (HPLC) coupled Electrospray Ionization tandem Mass Spectrometry (ESI/MS/MS) was employed in this study as a proven powerful technique to carry out intact anthocyanin and production-ion analysis. Anthocyanin compounds were identified by mainly matching MS fragments database (Giusti et al., 1999; Kim et al., 2012; Tian, Giusti, et al., 2005; Tian, Konczak, et al., 2005; Truong et al., 2010), HPLC retention time, and absorbance spectra pattern. The objective also intended to investigate cooking effects to anthocyanins and to reveal thermal-stable anthocyanins for future breeding purposes.

Materials and Methods

Chemicals: Acetonitrile, methanol, hexane, and formic acid were either HPLC grade or analytic grade purchased from Thermal Fisher Scientific (Suwanee, GA). Water used in all preparation and analysis was purified through Barnstead E-Pure Deionization System (Dubuque,

IA) and filtered using Millipore 0.45 µm membrane (Bedford, MA). Standards of cyanidin-3, 5-diglucoside was obtained from Sigma-Aldrich (St. Louis, MO).

Sample preparation: The anthocyanin source used in this study was purple-fleshed sweet potato (*Ipomoea batatas*) p40, a variety of anthocyanin-rich sweet potato cultured at the John C. Pair Horticulture Research Center, Wichita, KS. Intact root tubes of 120-150 g average weight were subjected to cooking conditions specified in Table 2. The cooked tubers were peeled, freeze-dried, and powdered using a mortar and pestle. An additional de-fatting procedure was performed on fried samples and the residual oil was believed to cause little interference in later analysis.

Table 2. Preparation and cooking conditions

Cooking methods	*Conditions	Equipment	Preparation
Baking	205 °C (400 °F), 50 min	Conventional oven	Manually selected and washed medium-size tubes. Injured, skunked, and spouted tubes were discarded.
Steaming	100 °C, 20 min	Hamilton Beach rice cooker w/ steaming sleeve	
High pressure cooking	121 °C, 15 psi, 17 min	Cuisinart pressure cooker	
Microwave baking	100% power, 5 min	Conventional 850 W microwave	
Frying	177 °C (350 °F), 5 min	Conventional deep fryer	Cut to ¼ inch thick wedges

* Conditions were based on conventional receipts and preliminary studies that determined fully cooking conditions.

Proximate Analysis: Lyophilized p40 powder were used for analyses of moisture, crude protein, lipid, and ash. Briefly, moisture was determined by hot oven method at 130 °C for two hours; protein was determined by a Leco FP-2000 protein analyzer (Leco Corp, St Joseph, MI, USA) using AOAC method 992.15 and 6.25 was used as the converting factor (King-Brink &

Sebranek, 1993); crude lipid and moisture was determined with a CEM Smart Trac system (CEM Corporation, Matthews, NC, USA) by AOAC method 2008.06 (Leffler et al., 2008); and ash content was determined using a muffle furnace by AOAC method 942.15 (Thiex, Novotny, & Crawford, 2012).

Extraction and Quantification: Defatting. Powdered fried PSP was extracted in hexane at solid to solvent ratio (1:6, w/v) for one hr, and centrifuged (3000g, 20 min, 4 °C). The procedure was repeated three times and the pellet was air dried overnight.

Anthocyanin Extraction. The extraction and subsequent analysis followed method of Kim et al., (2012), with modifications. One gram of powder containing internal standard, cyanidin-3, 5-diglucoside, was extracted with 20 mL 5% formic acid water on an orbital shaker incubated at 40 °C for 12 h and centrifuged (4000g, 20 min, 4 °C). The extraction was repeated and the supernatants were pooled. A Waters Sep-Pak C₁₈ solid phase extraction cartridge (Milford, MA) was activated with 3 mL methanol and 3 mL water, and loaded with 2 milliliters of supernatant. Anthocyanins were cleaned with 3 mL water and eluted with 1 mL methanol, and the cartridge should be free of visible color after elution. The eluent was evaporated in an Eppendorf Vacufuge (Hamburg, Germany) to dryness and re-constituted in 1 mL 5% formic acid water.

High-Performance Liquid Chromatography- Diode Array Detector (HPLC-PDA)

Analysis: Instruments. A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic separation. This system employed a DGU-20A3 built in degasser, a LC-20AB solvent delivery pump, a SIL-20AHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detectors. A Waters (Milford, MA) C₁₈ reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using LC Solution software (Kyoto, Japan).

Conditions. Elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in acetonitrile); gradient expressed as mobile phase B volume was 15-30% for 30 min, 30-50% in following five min and held at 50% for 10 min before returning to 15%. The flow rate was maintained as 1 mL/min and column temperature was 25 °C. Detector performed a full spectrum scan between 190-800 nm where 525 nm was used for monitoring anthocyanins. Cyanidin-3, 5-diglucoside was used for quantitation of anthocyanin peaks and as internal standard.

Mass Spectrometry Analysis: Mass spectrometric scan was performed in positive mode with a scanning interval 200-1200 m/z. Nebulization were conducted at 350 °C aided by concurrent N₂ flow at 10 psi; capillary and cone voltages were set at 3.5 kV and 40 V; drying gas flow rate was 5 L/min. Mass of precursor ions and reactions of fragments loss were evaluated. Data was analyzed using BrukerHystar Post Processing software (Bruker, Coventry, United Kingdom).

Acid Hydrolysis: Acid hydrolysis procedure was adopted from Truong et al., 2010. A 50 µL of anthocyanin extract was mixed with same volume 6 N HCl in a sealed HPLC vial. The mixture was heated in boiling water bath for 2 h after thorough vortex. Sample was cooled in ice bath and dried in an Eppendorf Vacufuge before re-constituted in 500 µL water acidified with 5% formic acid. Mass spectrometric scanning was performed at 100-800 m/z range to identify anthocyanidins.

Statistical Analysis: The anthocyanin change was analyzed by one-way ANOVA where cooking conditions were main factors. Tukey's post-hoc test was used to assess the multiple differences of individual anthocyanin at various cooking conditions. A probability of $P < 0.05$ was considered significant. Statistical procedures were handled by SAS software.

Results

Proximate analysis: Freeze drying reduced the moisture content of raw p40 flesh from 75% to 4.7%. Protein, fat, and ash contents were determined as 8.4%, 0.7%, and 4.3%, respectively. Thermal treatments resulted in leaching and drying effects and led to an impact on moisture contents of fresh sweet potato but did not alter the macronutrients after freeze drying.

Chromatographic Separation: As shown in Figure 5 and 6, anthocyanin eluents were separated under the experimental conditions. Twelve major peaks, possessed typical anthocyanin spectra of a maximum absorbance at around 520 nm, were further monitored with mass spectrometry. Figure 7 shows the representative spectra of peaks 1, 5, and 7. It is noted that absorbance peaks in ultraviolet range were also seen in most anthocyanins peaks, indicating an occurrence of acylations. Peaks 8, 9 and 10 were the major anthocyanins, which contributed to half of the total anthocyanin peak areas. Concentrations of the anthocyanins, along with their identities, were summarized in Table 3.

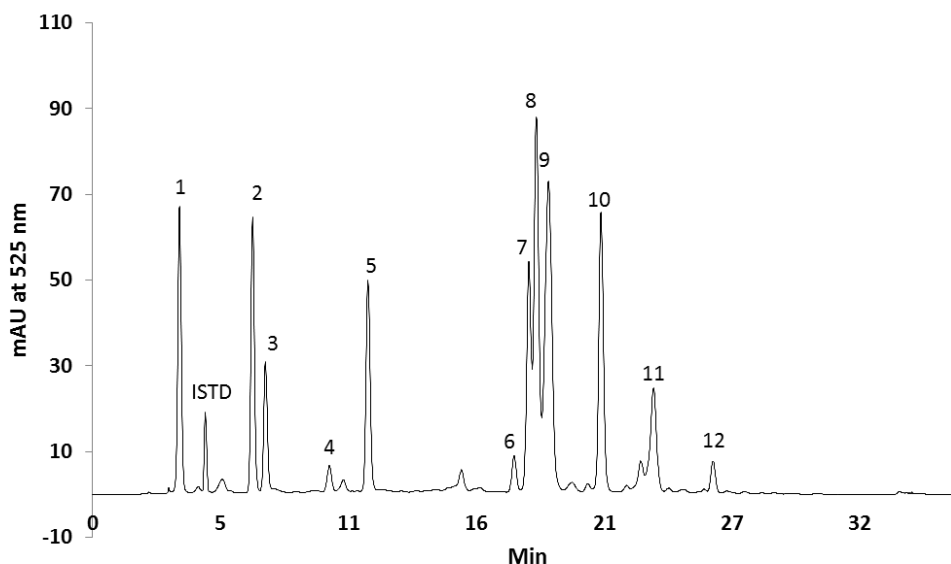


Figure 5. HPLC chromatogram of anthocyanin extract of raw p40

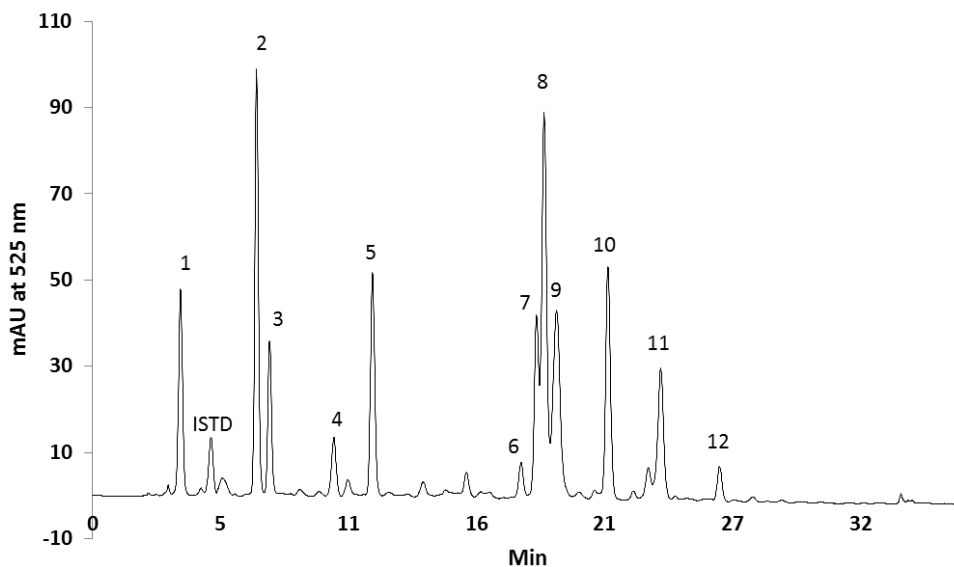


Figure 6. HPLC chromatogram of anthocyanin extract of baked p40

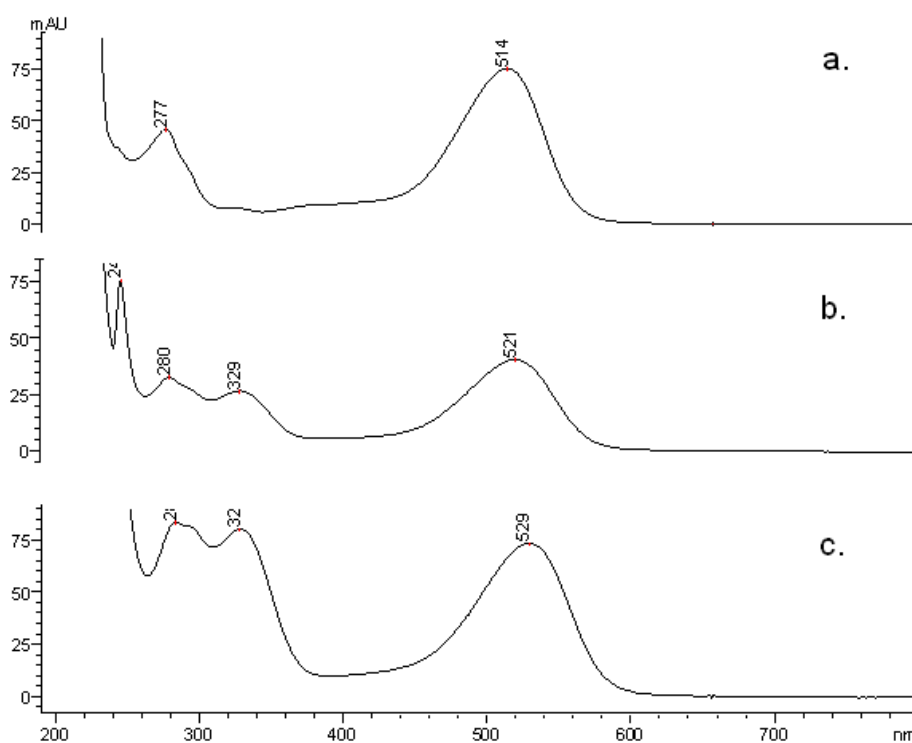


Figure 7. UV-vis spectra of the selected anthocyanin peaks: a: peak 1; b: peak 5; c: peak 7

Mass Spectrometric Identification: The m/z ratio of the intact anthocyanins were captured within the scanning interval ranging. Cyanidin (m/z 287) and peonidin (m/z 301) were

two aglycons detected. Glycosylations of glucose (m/z 162) and sophorose (m/z 324) were found linked to all 12 anthocyanins, with acylations of caffeic acid (m/z 180), ferulic acid (m/z 194), and p-hydroxybenzoic acid (m/z 138).

Table 3. Anthocyanin profile of p40

Peak number	Retention time (min)	Compound Identity	R1*	R2*	R3*	Parent ion, product ion (m/z)	% Peak area
1	3.618	Cyanidin 3-sophorose-5-glucoside	H	H	H	773, 611 449 287	9.25
2	6.668	Cyanidin 3-p-hydroxybenzoyl sophorose-5- glucoside	H	pHB	H	893, 731 449 287	8.83
3	7.203	Cyanidin 3-(6" -caffeoyl sophorose)-5-glucoside	H	Caf	H	935, 773 449 287	4.74
4	9.847	Peonidin 3-p-hydroxybenzoyl sophorose-5- glucoside	CH3	pHB	H	907, 745 463 301	1.29
5	11.484	Cyanidin 3-(6" -feruloyl sophorose)-5-glucoside	H	Fr	H	949, 787 449 287	8.09
6	17.582	Peonidin 3-(6" -feruloyl sophorose)-5- glucoside	CH3	Fr	H	963, 801 463 301	1.72
7	18.199	Cyanidin 3-(6" ,6" ' -dicaffeoyl sophorose)-5- glucoside	H	Caf	Caf	1097, 935 449 287	9.56
8	18.510	Cyanidin 3-caffeoyl-p-hydroxybenzoyl sophorose-5-glucoside	H	Caf	pHB	1055, 893 449 287	16.43
9	19.017	Peonidin 3-caffeoyl sophorose-5- glucoside	CH3	Caf	H	949, 787 463 301	19.72
10	21.209	Cyanidin 3-(6" -caffeoyl-6" ' -feruloyl sophorose)-5- glucoside	H	Caf	Fr	1111, 949 449 287	12.10
11	23.404	Peonidin 3-caffeoyl-p-hydroxybenzoyl sophorose-5-glucoside	CH3	Caf	pHB	1069, 907 463 301	6.32
12	25.884	Peonidin 3-(6" -caffeoyl-6" ' -feruloyl sophorose)-5- glucoside	CH3	Caf	Fr	1125, 963 463 301	1.53

*Refer to Figure 8 for the compound structures

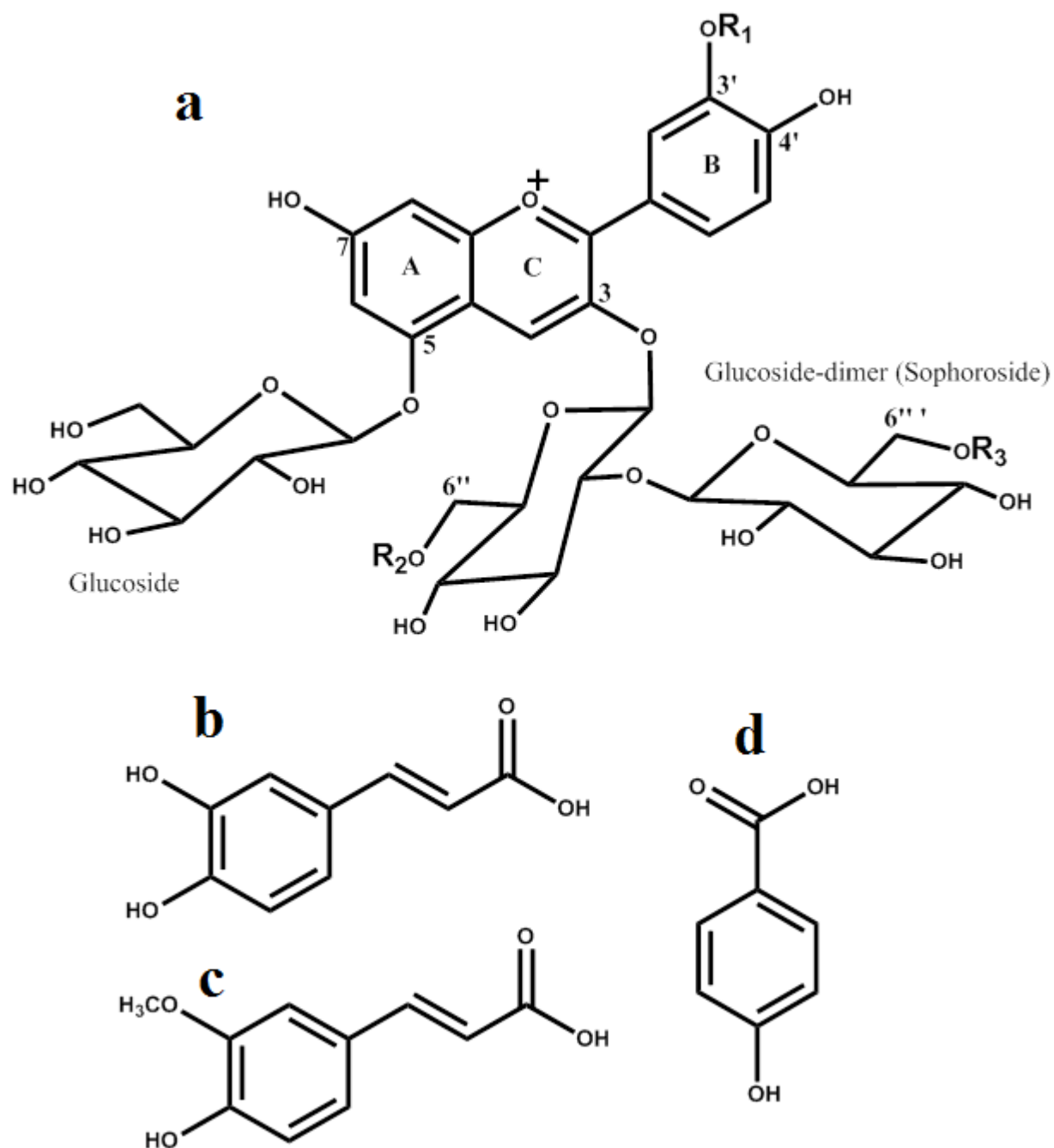


Figure 8. Skeleton structure of cyanidin ($R_1=H$) or peonidin ($R_1=CH_3$) 3-sophoroside-5-glucoside (a); b: caffeic acid (Caf); c: ferulic acid (Fr); d: p-hydrobenzoic acid (pHB).

Acid Hydrolysis: Acid hydrolysis greatly reduced the number of peaks to three. The 287 and 301 m/z were observed on MS1 spectra for peak 1 and 2, respectively, indicating they were

simple cyanidin and peonidin. Cyanidin and peonidin peaks in the hydrolysate of raw p40 were 82% and 8% of total peak area at 520 nm (Figure 9).

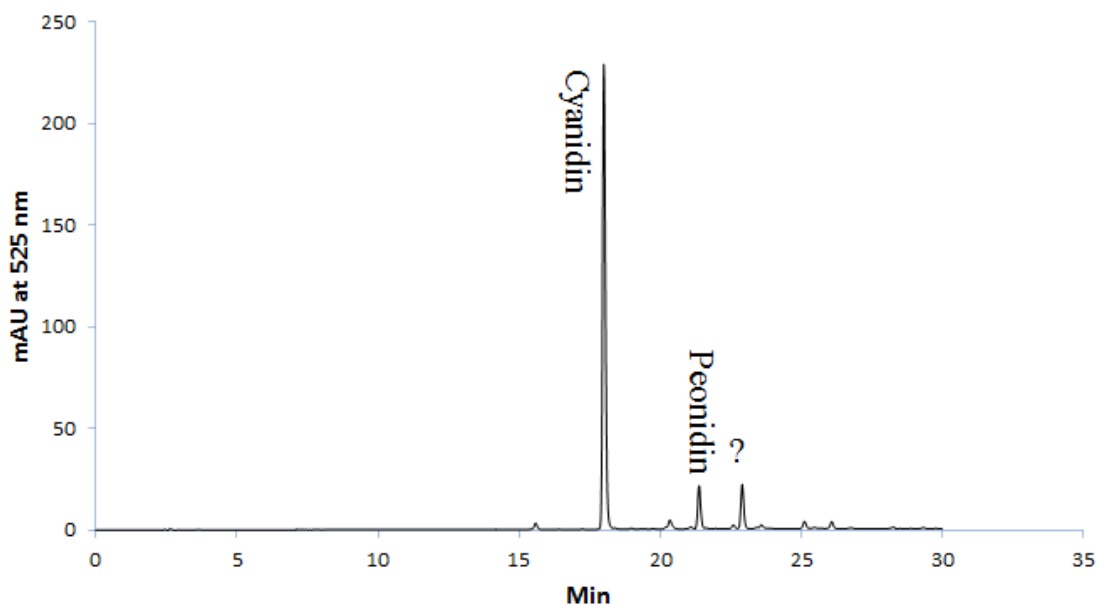


Figure 9. HPLC chromatogram of raw p40 hydrolysate

Effect of Thermal Treatments: Thermal treatments altered the anthocyanin distribution as shown in Figure 5 and 6. The stability of individual anthocyanin varied greatly. Later-eluted diacylated anthocyanins (peak 7-12) were more unstable than early-eluted ones (non-acylated, peak 2-6) and were reduced nearly half. Steaming, microwave baking, high-pressure cooking, and frying decreased the total anthocyanin content by 17.4%, 27.2%, 35.0%, and 21.7%, respectively (Table 4). Conventional baking was the only treatment that did not cause significant anthocyanin loss.

Table 4. Effect of thermal treatments to anthocyanin profile of p40

Compounds	mg/100g DW					
	Raw	Baked	Steamed	Microwave baked	High Pressure cooked	Fried
Cyanidin 3-sophoroside-5-glucoside	121±2.1 ^A	109±6.7 ^{AB}	122±7.4 ^A	88.5±3.1 ^{CD}	90.2±2.7 ^{BC}	69.5±4.2 ^D
Cyanidin 3-p-hydroxybenzoyl sophoroside-5- glucoside	115±0.6 ^C	191±1.5 ^B	186±2.2 ^B	305±15.2 ^A	172±2.7 ^B	136±8.6 ^C
Cyanidin 3-(6'' -caffeoyl sophoroside)-5-glucoside	62.0±1.6 ^{AB}	73.2±1.7 ^A	76.6±5.6 ^A	59.6±4.2 ^B	59.4±0.6 ^B	52.3±3.7 ^B
Peonidin 3-p-hydroxybenzoyl sophoroside-5- glucoside	16.7±1.7 ^D	37.2±0.6 ^B	29.8±0.9 ^C	71.9±3.3 ^A	26.7±0.1 ^C	18.5±1.5 ^D
Cyanidin 3-(6'' -feruloyl sophoroside)-5-glucoside	106±3.3 ^B	120±1.4 ^B	217±9.2 ^A	125±7.5 ^B	170±1.0 ^A	115±8.2 ^B
Peonidin 3-(6'' -feruloyl sophoroside)-5-glucoside	22.5±3.8 ^A	25.6±0.4 ^A	15.1±1.0 ^C	17.8±0.9 ^{BC}	17.5±0.8 ^{BC}	23.6±0.4 ^{AB}
Cyanidin 3-(6'' ,6'' ' -dicaffeoyl sophoroside)-5-glucoside	125±4.1 ^A	104±2.6 ^B	54.4±5.0 ^C	34.8±0.9 ^D	52.1±0 ^C	103±1.9 ^B
Cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside	215±26 ^A	235±1.8 ^A	102±8.3 ^B	125±1.3 ^B	129±5.2 ^B	218±7.5 ^A
Peonidin 3-caffeoyl sophoroside-5-glucoside	258±26 ^A	171±2.7 ^B	114±8.8 ^{CD}	70.2±1.2 ^D	110±4.9 ^{CD}	157±4.9 ^{BC}
Cyanidin 3-(6'' -caffeoyl-6'' ' -feruloyl sophoroside)- 5-glucoside	158±4.1 ^A	143±4.7 ^B	103±7.9 ^C	49.9±0.9 ^D	102±4.7 ^C	144±3.6 ^B
Peonidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside	82.6±24 ^{AB}	92.7±7.9 ^A	34.8±2.6 ^B	47.4±0.2 ^B	35.0±1.7 ^B	41.8±0.9 ^B
Peonidin 3-(6'' -caffeoyl-6'' ' -feruloyl sophoroside)-5-glucoside	20.0±1.9 ^A	23.0±0.2 ^A	15.1±1.3 ^B	10.8±0.1 ^C	14.6±0.7 ^{BC}	17.1±0.2 ^B
Total Anthocyanin	1282±97 ^A	1302±32 ^A	1055±59 ^B	995±39 ^B	934±24 ^B	1079±45 ^B

*data are expressed as mean ± SD (n=2) on dry basis. Values marked by different letters within same rows indicate significant differences (P<0.05)

Discussion

The major effort of this study focused on characterizing anthocyanin species in p40 and to evaluate thermal stability. HPLC-MS/MS analysis was applied for the methanolic extract of p40 before and after a number of thermal treatments and the anthocyanin contents were assessed.

Twelve anthocyanins were detected by HPLC. The mixture of p40 anthocyanins exhibited maximum absorbance around 520 nm (Figure 5 and 6). Cyanidin-3, 5-diglucoside was added in p40 as an internal standard. Previous studies have shown anthocyanin contents of PSP varieties varied greatly from 32 to 1342 mg/100g (Table 4). The total content of anthocyanins in p40 flesh was estimated 1282 mg/100g dry weight and was the second highest. When comparing p40 to berry fruits and colored vegetables, p40 (~3000 mg/kg fresh weight) ranked at upper-middle of the ladder and was superior to strawberry (350 mg/kg) and red cabbage (250 mg/kg), and was comparable to blueberry (4200 mg/kg), raspberry (4277 mg/kg), and cherry (4500 mg/kg) (Clifford, 2000).

Identification of anthocyanins was greatly relied on the matching fragment patterns to the anthocyanin library pooled from a number of published articles (Giusti et al., 1999; Kim et al., 2012; Otake, Terahara, Saito, Toki, & Honda, 1992; Tian, Giusti, et al., 2005; Tian, Konczak, et al., 2005; Truong et al., 2010). The m/z of the precursor ions were detected after electrospray ionization, and the precursor ions were further dissociated by argon collision. During the collision, the glycosidic bonds joining anthocyanidins and saccharides were vulnerable, and often cleaved; therefore, by matching residual fragmentation, the anthocyanins were positively identified. Ions of peak 1 (cyanidin 3-sophoroside-5-glucoside, m/z 773) produced abundant fragments of m/z 611, 449, and 287 after argon collision. Transition $773 > 611$ and $773 > 449$ represented the loss of glucose (m/z 162), and sophorose (m/z 324); transition $773 > 287$ produced cyanidin (m/z 287) aglycon resulted from loss of both glucose and sophorose (Figure 10). Mono-

and di-acylated anthocyanins exhibited somewhat similar fragmentation patterns. As shown in Figure 11, transitions of m/z 893 (cyanidin 3-*p*-hydroxybenzoyl sophoroside-5- glucoside) to 731, 449, and 287 were observed, where $893 > 449$ transition indicated the loss of sophoroside and acylation. Identifications of the rest anthocyanins were carried out in the similar fashion. Tandem MS exhibited its unique advantage of distinguishing isomers. For example, cyanidin 3-(6'' -feruloyl sophoroside)-5-glucoside (peak 5) and peonidin 3-caffeoyl sophoroside-5-glucoside (peak 9) both had similar molecular weight (m/z 949), and their identities were revealed by subsequent fragmentation.

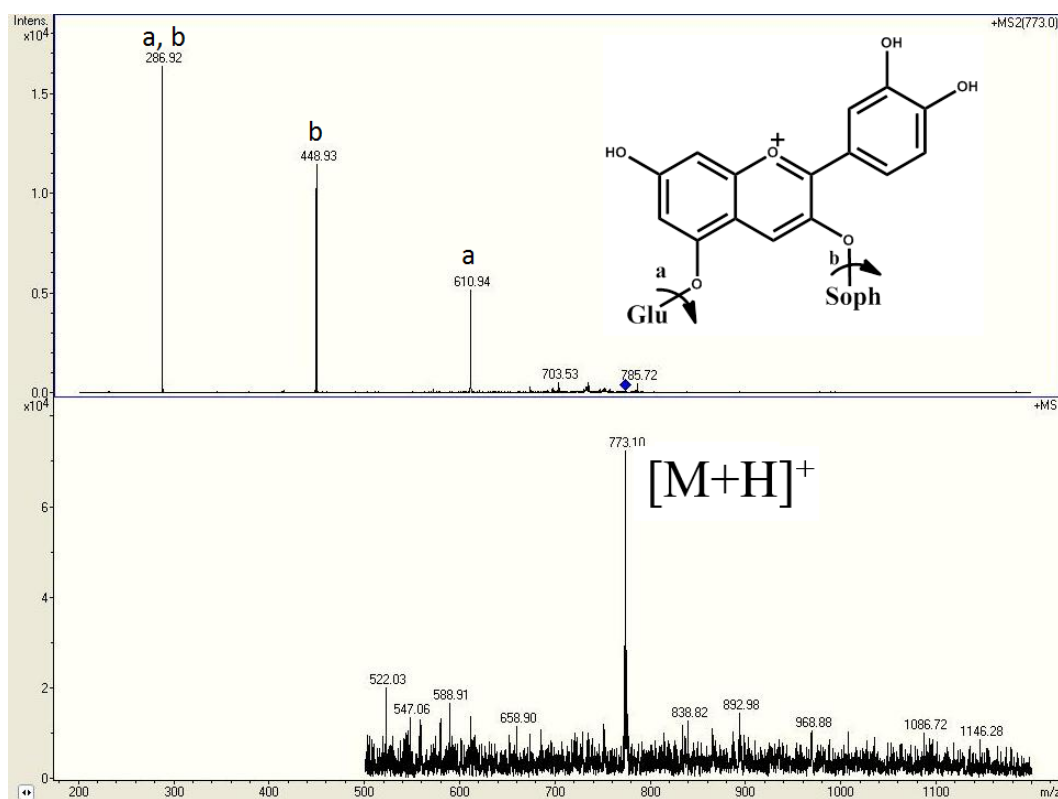


Figure 10. Mass spectra of cyanidin 3-sophoroside-5-glucoside; a, b: bond cleavage

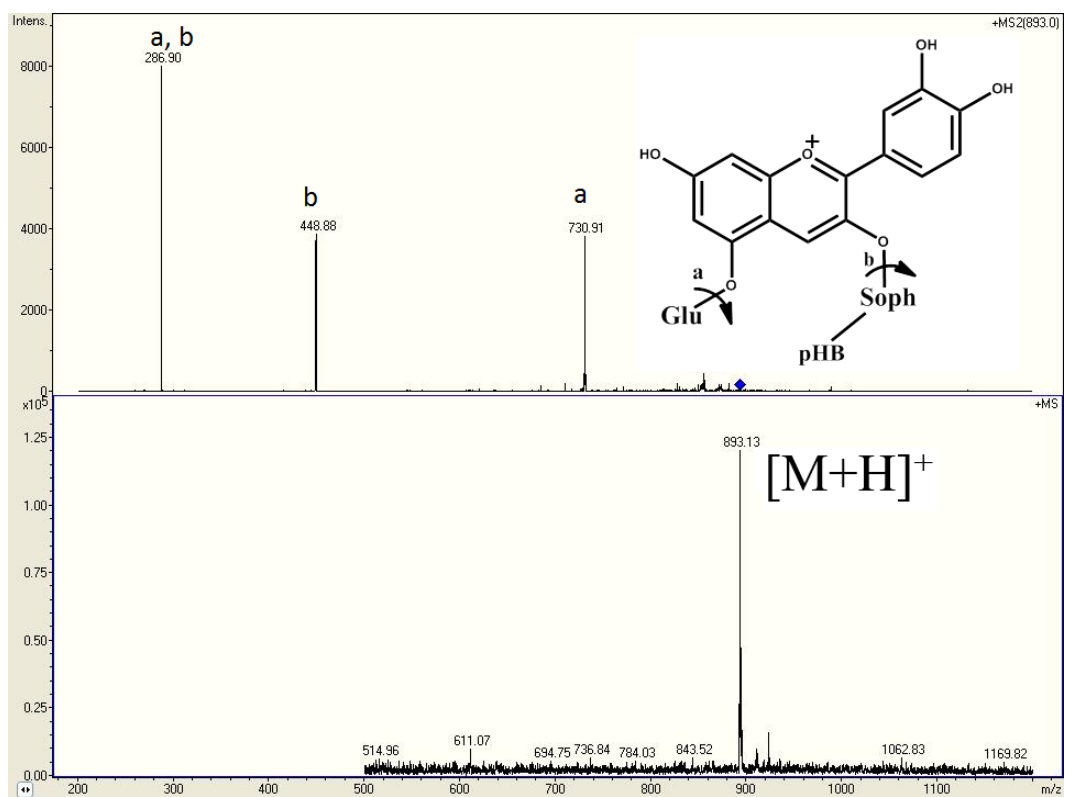


Figure 11. Mass spectra of cyanidin 3-p-hydroxybenzoyl sophoroside-5- glucoside; a, b: bond cleavage

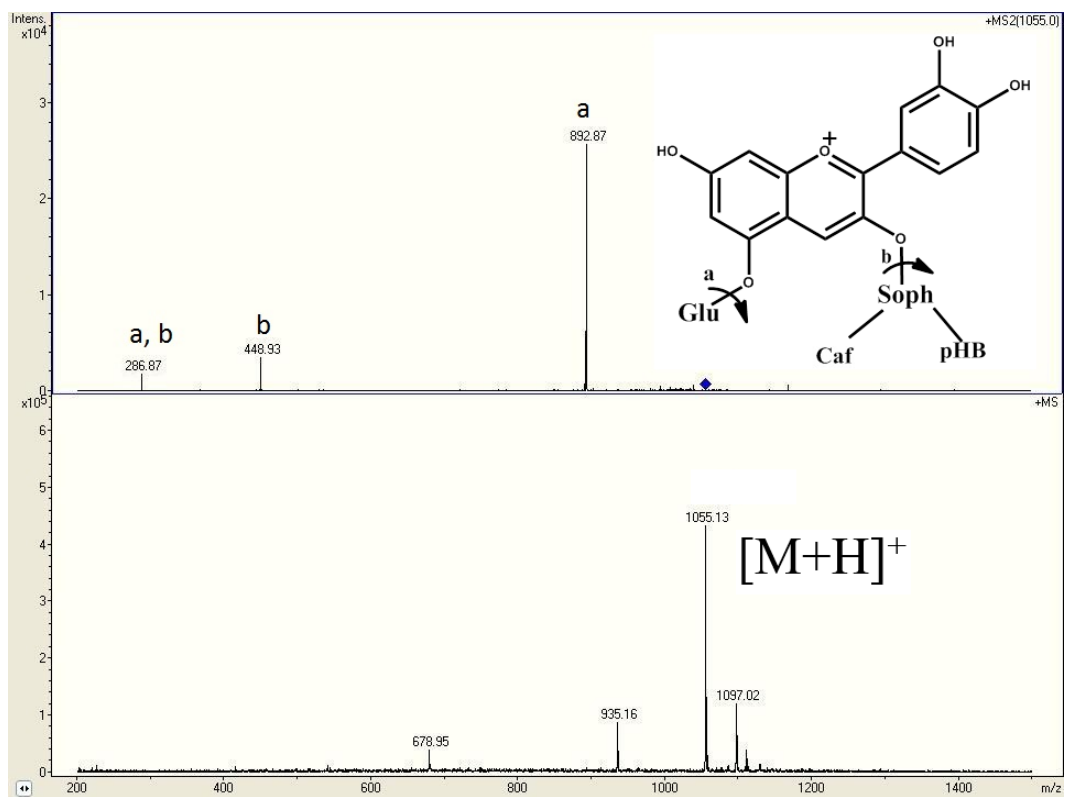


Figure 12. Mass spectra of cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside; a, b: bond cleavage

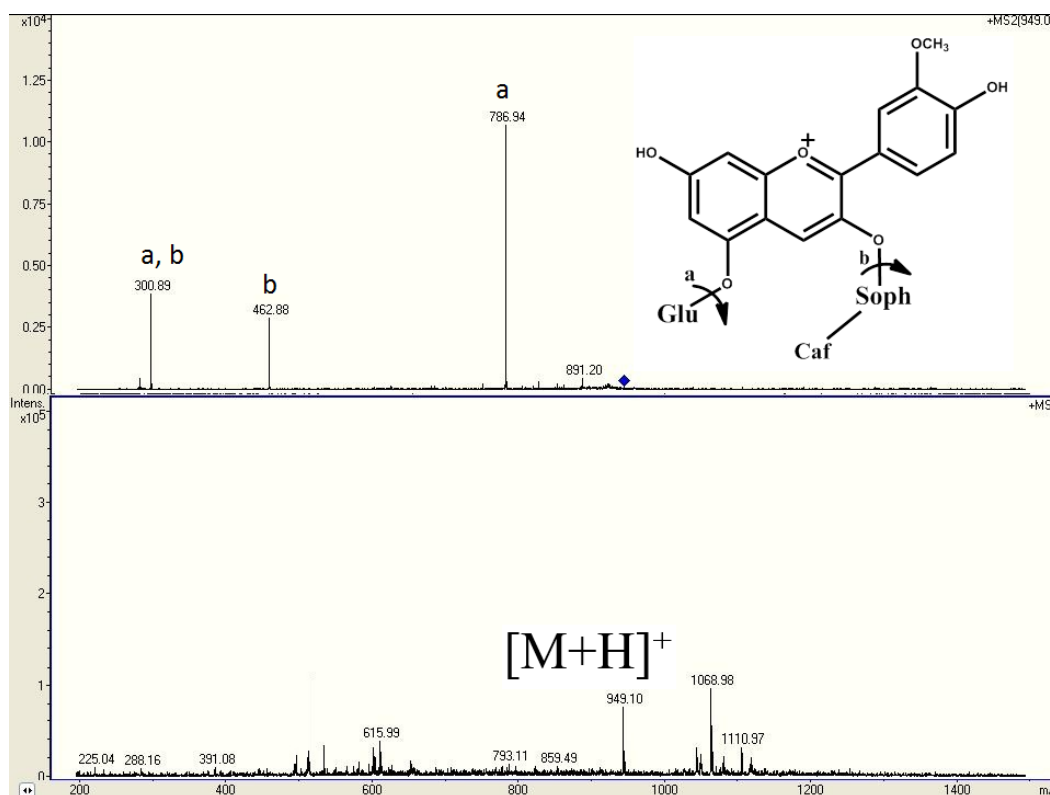


Figure 13. Mass spectra of peonidin 3-caffeoyl sophorose-5-glucoside; a, b: bond cleavage

PSPs are classified to either cyanidin or peonidin type based on their peonidin/cyanidin ratio (pn/cy). Pn/cy is an important factor to flesh color and may present difference in functionalities. Peonidin type (pn/cy>1) sweet potatoes generally have a pink to red flesh color and they sometimes are referred as red-fleshed. As content of cyanidin compounds increase, the color of the flesh shifts to purple and dark purple. Structure-wise, cyanidin contains more hydroxyl groups than peonidin, leading to higher antioxidative activity (Yoshimoto et al., 1999), reported cyanidin type pigments were superior to peonidin in antimutagenicity and were desired due to the intensity of the color. However, as to year 2001, no variety with high content of cyanidin type compound has been developed (Table 5) (Montilla, Hillebrand, & Winterhalter, 2011; Yoshimoto et al., 1999; Yoshimoto, Okuno, Yamaguchi, & Yamakawa, 2001). From HPLC analysis, p40 was classified as a cyanidin-type PSP (pn/cy 0.45) with exceptionally high

total anthocyanin. To further confirm dominating anthocyanin type, acid hydrolysis test was conducted. Acid hydrolysis changed the retention and consolidated the number of peaks to three. Eluent at 18.08, 21.51 min were confirmed as simple cyanidin and peonidin, where pelargonidin, delphinidin, petunidin and malvidin were not found in p40. It appeared the chosen acidity at the boiling temperature completely hydrolyzed p40 anthocyanins to aglycons. Acid hydrolysis is a rapid and accurate method to investigate anthocyanidin ratio without a costly mass spectrometry instrument, and more importantly, it consolidates many raw anthocyanin chromatograms to avoid co-eluting. The objective of acid hydrolysis--to confirm anthocyanidin composition in addition to mass spectrometric results--was achieved. Taken together with MS/MS results, p40 was positively a cyanidin-type PSP with the highest anthocyanin content.

Table 5. Selected data of previous studies of anthocyanin-rich sweetpotatoes

Variety name	Anthocyanin mg/100g DW	Pn/cy	Method, standard	Reference
Stokes Purple	328*	4.0	pH differential, cy 3-glu	Truong et al., 2010
NC 415	178*	2.0		
Okinawa	65*	0.1		Kim et al., 2012
Shinzami	1342	3.4	HPLC, cy 3, 5- diglu	Suda et al., 2003;
AyaMurasaki	261*	3.9	HPLC, pn 3- caf•sop-5-glu	Yoshimoto et al., 2001; Yoshinaga,
TanegashimaMurasaki	32**	0.28	Not reported	Yamakawa, & Nakatani, 1999
12 varieties PSP bred in central China	78-695	0.96-1.1	HPLC, cy 3-glu	Zhu, Cai, Yang, Ke, & Corke, 2010
NDOP5847-1 (red- fleshed potato)	175	No cyanidin	pH differential, pg-3-glu	Rodriguez-Saona, Giusti, & Wrolstad, 1998
p40	1282	0.45	HPLC, cy 3, 5- diglu	

*Values converted from fresh weight basis to dry basis using water%=77 (The USDA Food and Nutrient Database for Dietary Studies); **Calculated from statements “TanegashimaMurasaki is a cyanidin-type variety, but the content of anthocyanin pigment is about one eighth that of Ayamurasaki (41)”;

cy: cyanidin; pn: peonidin; glu: glucoside; caf: caffeoyl; sop: sophoroside; pg: pelargonidin

There was a significant change on anthocyanin profile of p40 after thermal treatments. Total anthocyanin contents, in equivalence of cyanidin-3, 5-diglucoside were reduced from 1282 to 1055, 995, 934, and 1079 mg/100 g after been steamed, microwave baked, high pressure cooked, and fried, respectively. However, conventional baking best preserved anthocyanins and the total anthocyanin content showed no significant difference to raw. Interestingly, reduction did not occur proportionally in every anthocyanin compound. As a trend observed, di-acylated anthocyanins were more susceptible to heat and their contents decreased 2-3 folds; on the other hand, mono-acylated ones had minimum loss and some even showed tendency to increase. In the microwave baking group, cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside increased from 115 to 305 mg/100 g, where cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside was reduced from 215 to 125 mg/g. It is possible that thermal treatments have caused more anthocyanin to be released from physical entrapment in other structures (Xu & Chang, 2008b). Peonidin 3-p-hydroxybenzoyl sophoroside-5-glucoside (peak 4) was increased significantly as well after most treatments, and its content was elevated to 7.2% of total anthocyanin from 1.3% after microwave baking. As a subgroup of polyphenolic pigment, anthocyanins are expected to be degraded by heat like many structurally similar flavones and phenolic acids (Xu & Chang, 2008a). It appeared that p40 anthocyanins varied greatly in their thermal stabilities, and most likely acylation played a role of stabilizing anthocyanins in heat. Anthocyanins mono-acylated with p-hydroxybenzoic acid was best retained after heating, followed by ferulic acid and caffeic acid. The losses of total peonidin and cyanidin derivatives were approximately 40% and 12%, respectively, after most treatments. Another trend observed was shifting of late eluted peaks to early eluted peaks, which have happened in every chromatogram, suggested di-acylation

compounds were destroyed to greater extent. Possible explanations were given as di-acylation compound transformed and strengthened mono-acylated compounds. For example, peak 10 to peak 5 is a loss of caffeic acid. Furthermore, thermal treatments may have better released phytochemicals by destroying and softening bound food matrices (Xu & Chang, 2009). Kim et al., (2012) reported anthocyanins in PSP Shinzami were reduced by 19% and 44% after roasting and steaming, respectively. We observed the similar trend at less severity in p40, probably because p40 has a very different anthocyanin profile.

Taken together, HPLC/MS/MS as a powerful way to fingerprint the anthocyanin profile has been applied in p40. Total 12 anthocyanins combining of mostly di- and mono-acylated cyanidin and peonidin have been identified. Caffeic acid, p-hydroxybenzoic acid, and ferulic acid are the major acylations and they are found attached to hexose sugar on anthocyanidin aglycons. To the best of our knowledge, p40 seems the first cyanidin-type purple sweetpotato with superior anthocyanin content up to 13 mg/g. While conventional baking did not reduce anthocyanin content significantly, other thermal treatments facilitated anthocyanin degradation and shifted the anthocyanin profiles in p40. The p-hydroxybenzoic acid appeared better stabilizing power than other acyl groups. Cyanidin 3-p-hydroxybenzoyl sophoroside-5-glucoside, peonidin 3-p-hydroxybenzoyl sophoroside-5- glucoside, and cyanidin 3-(6" -feruloyl sophoroside)-5-glucoside showed tendencies to increase after thermal treatment, which warrants further studies.

Conclusion

Twelve individual anthocyanins were identified and quantified in the newly bred purple sweetpotato p40, where seven were cyanidin derivatives and five were peonidin derivatives. Our result showed p40 is a cyanidin type PSP (pn/cy ratio 0.45) with exceptionally high anthocyanin

content up to 13 mg/g. Acid hydrolysis test confirmed cyanidin was presented at predominating amount. This is the first time a high cyanidin PSP variety was documented. Cyanidin and peonidin 3-sophoroside-5-glucoside were identified as the base structure of p40 anthocyanins, where caffeic acid, p-hydroxybenzoic acid, and ferulic acid were found as acylations. Thermal treatments significantly altered anthocyanin profile, di-acylated anthocyanins appeared to degrade and strengthen mono-acylated anthocyanins, which partially contribute to the increase of mono-acylated anthocyanins. The results show that p-hydroxybenzoic acid had the best stabilizing force, but there should be further studies on this topic to benefit future breeding.

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